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Epstein-Barr Virus EBNA1 Protein Regulates Viral Latency through Effects on let-7 MicroRNA and Dicer

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ABSTRACT

The EBNA1 protein of Epstein-Barr virus (EBV) plays multiple roles in EBV latent infection, including altering cellular pathways relevant for cancer. Here we used microRNA (miRNA) cloning coupled with high-throughput sequencing to identify the effects of EBNA1 on cellular miRNAs in two nasopharyngeal carcinoma cell lines. EBNA1 affected a small percentage of cellular miRNAs in both cell lines, in particular, upregulating multiple let-7 family miRNAs, including let-7a. The effects of EBNA1 on let-7a were verified by demonstrating that EBNA1 silencing in multiple EBV-positive carcinomas downregulated let-7a. Accordingly, the let-7a target, Dicer, was found to be partially downregulated by EBNA1 expression (at the mRNA and protein levels) and upregulated by EBNA1 silencing in EBV-positive cells. Reporter assays based on the Dicer 3′ untranslated region with and without let-7a target sites indicated that the effects of EBNA1 on Dicer were mediated by let-7a. EBNA1 was also found to induce the expression of let-7a primary RNAs in a manner dependent on the EBNA1 transcriptional activation region, suggesting that EBNA1 induces let-7a by transactivating the expression of its primary transcripts. Consistent with previous reports that Dicer promotes EBV reactivation, we found that a let-7a mimic inhibited EBV reactivation to the lytic cycle, while a let-7 sponge increased reactivation. The results provide a mechanism by which EBNA1 could promote EBV latency by inducing let-7 miRNAs.

IMPORTANCE

The EBNA1 protein of Epstein-Barr virus (EBV) contributes in multiple ways to the latent mode of EBV infection that leads to lifelong infection. In this study, we identify a mechanism by which EBNA1 helps to maintain EBV infection in a latent state. This involves induction of a family of microRNAs (let-7 miRNAs) that in turn decreases the level of the cellular protein Dicer. We demonstrate that let-7 miRNAs inhibit the reactivation of latent EBV, providing an explanation for our previous observation that EBNA1 promotes latency. In addition, since decreased levels of Dicer have been associated with metastatic potential, EBNA1 may increase metastases by downregulating Dicer.

Epstein-Barr virus (EBV) is a gammaherpesvirus that infects most people worldwide and is associated with several types of B-cell lymphomas, as well as nasopharyngeal carcinoma (NPC) and gastric carcinoma (1, 2). The EBV life cycle consists of both latent and lytic modes of infection in B lymphocytes and epithelial cells. Although EBV mainly exists in a latent mode of infection in B cells, it occasionally reactivates to the lytic state for cell-to-cell spread. In addition, lytic reactivation of EBV in epithelial cells of the orthopharynx is necessary for the production of viral particles required for host-to-host transmission of the virus. Lytic infection begins with the expression of BZLF1 (or Zta), followed by the expression of BRLF1 (or Rta). Together these proteins activate the cascade of subsequent lytic gene expression and enable the generation of linear viral genomes for packaging (3). Abortive lytic infections in which BZLF1 is expressed in the absence of late lytic proteins have also been reported, and these appear to be important for EBV-induced cancers (4, 5).

EBV latent infection involves the expression of a small subset of EBV proteins and immortalization of the infected cells. Epstein-Barr nuclear antigen 1 (EBNA1) is the only EBV protein expressed in immortalized cells in all types of latent infection and, in one form of latency, is the only viral protein expressed (6). EBNA1 is the only EBV protein required to replicate and segregate the EBV episomal genomes in latency, resulting in the maintenance of the EBV genomes at a stable copy number (7, 8). These functions require EBNA1 binding to the latent origin of replication, *oriP* (8). In addition, EBNA1 binding to the family of repeat (FR) sequences within *oriP* can transactivate the expression of other EBV latency genes (9, 10). The transactivation activity of EBNA1 has been mapped to two EBNA1 regions: amino acids 61 to 83 in the N terminus and an internal Gly-Arg-rich sequence (amino acids 325 to 376) which is also essential for segregation function (11–13). The transcriptional activation activity of the region from amino acids 61 to 83 appears to involve an interaction with the acetylated histone reader protein Brd4 (14), while transactivation by the Gly-Arg sequence involves interactions with the related nucleosome assembly proteins, NAP1, TAFI-β, and nucleophosmin (15–17).

In addition to its functions at EBV episomes, EBNA1 affects the cellular environment in multiple ways that contribute to EBV persistence and cell survival (18, 19). For example, EBNA1 counter-

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acts the stabilization of p53 by USP7 and induces the loss of PML nuclear bodies through the degradation of PML proteins, both of which contribute to the ability of EBNA1 to interfere with apoptosis and DNA repair (20, 21). EBNA1 has also been found to inhibit transforming growth factor β and NF-κB signaling and to induce oxidative stress (22–25). EBNA1 may also be able to transactivate the expression of some cellular genes. Indeed, microarray and chromatin immunoprecipitation experiments indicated that EBNA1 upregulates a small proportion of cellular genes and associates with their promoter regions, consistent with direct transactivation of these genes (26). In addition, EBNA1 was shown to induce transcripts encoding survivin through interactions with Sp1 at the survivin promoter (27). This effect was abrogated by deleting the EBNA1 N-terminal transactivation sequence, suggesting a transactivation mechanism similar to that which occurs within the EBV genome (27).

MicroRNAs (miRNAs) also contribute to EBV infection and associated cancers in a variety of ways (28, 29). MicroRNAs are noncoding RNA molecules that are mainly transcribed as long primary transcripts (pri-miRs) by RNA polymerase II (30). The pri-miRs are first processed into 60- to 70-nucleotide-long precursor miRNAs (pre-miRs) by a complex containing the RNase III enzyme Drosha and double-stranded RNA-binding protein DGCR8 (31). These pre-miRs are transported out of the nucleus and then further processed into mature 21- to 25-nucleotide-long miRNAs by a complex of Dicer (another RNase III enzyme), the TRPB double-stranded RNA binding protein, and an Argonaute protein (31, 32). Once loaded onto the Argonaute protein, the mature miRNAs bind to the 3′ untranslated region (UTR) of mRNAs through a highly conserved seed sequence (nucleotides 2 to 8), thereby inhibiting translation or enhancing degradation of the mRNA molecules (31). Cellular miRNAs regulate many processes, including cell survival and proliferation, in part by mediating the actions of tumor suppressors and signaling pathways (33); for example, p53 and ATM activation has been shown to impact specific miRNAs (34–36). As a result, aberrant patterns of expression of particular miRNAs are associated with specific cancers (33). The action of EBV proteins can also involve miRNAs. EBV LMP1, which contributes to cell transformation, has been shown to affect the levels of induction of several miRNAs, including miR-146a (37). Similarly, the EBV latency protein EBNA2 alters the levels of some cellular miRNAs, in particular, upregulating miR-21 and downregulating miR-146a (38). Cellular miRNAs can also affect the balance between EBV latent and lytic infection. For example, miR-200b and miR-429 were found to induce reactivation of latent EBV to the lytic cycle by downregulating the expression of the cellular ZEB1 and ZEB2 proteins that repress BZLF1 expression (39). EBV itself contains 44 miRNAs targeting both cellular and EBV mRNAs and contributing in multiple ways to cell survival and the regulation of EBV gene expression (29, 40).

In this study, we investigated whether EBNA1 alters the cellular miRNA profile in NPC cell lines. We show that EBNA1 expression affects specific miRNAs and, in particular, upregulates multiple members of the let-7 miRNA family. Further experiments confirmed that EBNA1 consistently induced let-7a miRNA and downregulated the let-7a target protein Dicer in NPC and gastric carcinoma cell lines and that these changes inhibited EBV lytic reactivation. The results suggest an additional function for let-7a miRNAs in regulating EBV reactivation and suggest a mechanism by which EBNA1 contributes to the maintenance of EBV latency.

MATERIALS AND METHODS

Cell lines. C666-1 cells are EBV-positive NPC cells (41). AGS-EBV cells are AGS gastric carcinoma cells containing Akata EBV with a neomycin resistance cassette (42). C666-1 and AGS-EBV cells were cultured in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum. HONE-Akata cells are HONE1 NPC cells infected with Akata EBV containing neomycin resistance and green fluorescent protein cassettes (43, 44). CNE2Z and HONE1 are NPC cells that have lost the EBV genome (45). CNE2Z, HONE1, and HONE1-Akata cells were grown in alpha-MEM (Gibco) supplemented with 10% fetal calf serum. For HONE-Akata and AGS-EBV cells, G418 (400 μg/ml; Life Technologies) was included in the medium to select for cells containing the recombinant EBV genome.

Generation and Illumina sequencing of small RNA libraries. HONE1 and CNE2Z cells were seeded in 10-cm dishes and transfected 24 h later with 6 μg pc3EBNA1 (12) or pcDNA3 (Invitrogen) and 2 μg pcYFP (provided by J. McDermott, York University [46]) using the Lipofectamine 2000 reagent (Life Technologies). Transfection efficiency was assessed by immunofluorescence microscopy using the EBNA1-specific polyclonal antibody R4 (17) and by microscopy for yellow fluorescent protein (YFP)-positive cells. Coexpression of both YFP and EBNA1 was confirmed in most cells. Approximately 5×10^6 cells were sorted for YFP-positive cells 24 h following transfection using a FACSCalibur flow cytometer (Becton Dickinson; University of Toronto Flow Cytometry Facility). Total RNA was isolated from 2×10^6 cells, followed by small RNA library construction as described previously (47), with the exception that a 4-nucleotide bar code sequence was added to the 3′ end of the 5′ linker for each sample and the samples were multiplexed. The following bar code sequences were used: AGTC for CNE2Z cells and EBNA1 replicate 1, TCAG for CNE2Z cells and EBNA1 replicate 2, CTGA for CNE2Z cells and pcDNA replicate 1, GTCA for CNE2Z cells and pcDNA replicate 2, GACT for HONE1 cells and EBNA1 replicate 1, TGAC for HONE1 cells and EBNA1 replicate 2, CAGT for HONE1 cells and pcDNA replicate 1, and CATG for HONE1 cells and pcDNA replicate 2. Briefly, RNA of less than 200 nucleotides was extracted from 100 μg of total RNA using a mirVana miRNA isolation kit (Ambion, Austin, TX) following the manufacturer's protocol. Small RNAs of ~18 to 30 nucleotides were then isolated by gel extraction, and libraries were generated for Illumina sequencing by sequential ligation of 3′ and 5′ adapters to the small RNA samples. cDNA was then synthesized (using SuperScript III reverse transcriptase [Life Technologies]) and PCR amplified (using *Ex Taq* DNA polymerase [TaKaRa]), and the amplified fragments, ranging from 110 to 130 bp in length, were gel extracted. High-throughput sequencing of the small RNA libraries was performed using the Illumina HiSeq2500 platform (Donnelly Sequencing Centre, University of Toronto). Custom Perl scripts were used to analyze the Illumina sequencing data. Briefly, total reads (~391 million) were first binned according to the 5′ bar code sequences that distinguished each sample. The reads were then trimmed to remove the 5′ bar code and 3′ linker sequences. The small RNAs were then aligned to the set of EBV and human miRNAs (downloaded from the miRBase database, release 18 [48]) using the BLAT tool (49). Only reads with perfect matches that were at least 17 nucleotides were considered. The set of reads matching miRNAs was examined for their size and first nucleotide distribution. miRNAs were determined to be altered by comparing the number of reads for a particular miRNA to the total number of miRNAs per library.

Transfections and transductions for let-7a experiments. Approximately 2×10^5 cells of the indicated cells in a 10-cm dish were transfected with 6 μg pc3EBNA1 or pc3DNA using Lipofectamine 2000 (Life Technologies). For the experiments whose results are presented in Fig. 3, EBNA1 expression plasmid pc3oriPEBNA1 or pc3oriPΔ61-83 was used, and the results were compared to those obtained with pc3oriP as a negative control (11). EBNA1 was overexpressed in AGS-EBV cells using an adenovirus delivery system. AGS-EBV cells (5×10^5) were incubated with adenovirus expressing SPA-tagged EBNA1 or SPA-tagged β-galactosidase (β-Gal) as a negative control (as described previously [50]), cells were harvested 48 h following infection, and the expression of EBNA1 and

β-Gal was verified by immunofluorescence microscopy. For EBNA1 silencing experiments, approximately 1.5×10^5 C666-1, AGS-EBV, or HONE-Akata cells plated in 6-cm dishes were transfected with 100 pmol of small interfering RNA (siRNA) against either EBNA1 (GGAGGUUCC AACCCGAAAUTT) or AllStar negative-control siRNA (Qiagen), using 2 μl of Lipofectamine 2000 (Life Technologies). The cells were subjected to second and third rounds of the same transfection after 24 and 48 h. For C666-1 cells, siRNA transfections were repeated at 48 and 96 h following the first round of transfection. Cells were harvested for RNA extraction or Western blotting at 24 h posttransfection or postransduction or 24 h after the last siRNA treatment.

PCR-based quantification of miRNA and primary RNA (pri-RNA). Total RNA was isolated from snap-frozen cell pellets using the TRIzol reagent (Life Technologies). The quantity and quality of the extracted RNA were determined by reading the optical densities at 260 and 280 nm (OD_{260} and OD_{280}, respectively) using a NanoDrop spectrophotometer (Thermo Scientific). For mRNA quantification, total RNA (1 μg) was reverse transcribed in a 25-μl reaction mixture using SuperScript III reverse transcriptase (Life Technologies) and random hexamer primers according to the manufacturer's instructions. Quantitative real-time PCR was performed using 0.2 μl of the cDNA and SsoFast EvaGreen Supermix (Bio-Rad) on a Rotorgene quantitative PCR system (Corbett Research). Primers used for mRNA quantification were the following: pri-let-7afd forward (5′-GAAACCTTTTGCTTCTTGTCT-3′) and pri-let-7afd reverse (5′-CCTCACTCTGATAGAGCAAT-3′), pri-let-7a-2 forward (5′-A TACTGAATCCCTCAAAGCC-3′) and pri-let-7a-2 reverse (5′-GAAAG GTAAGATTGGGTACGA-3′), and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) forward (5′-CTCCAAACAGCCTTGCTTGCTTCGAGA ACCATTTGCTTCCCGCTCAGACGTCTTGAGTGCTACAGGAAGCT GGCA-3′) and GAPDH reverse (5′-GACCCGACCCCAAAGGCCAGGC TGTAAATGTCACCGGGAGGATTGGGTGTCTGGGCGCCTCGGGG AACCTGCCCT-3′). For let-7a, let-7g, miR-92a, and BART6 miRNA and U6 snRNA quantification, 20 ng of total RNA was reverse transcribed using SuperScript III reverse transcriptase and measured by real-time PCR using TaqMan miRNA assays (Applied Biosystems) according to the manufacturer's protocol. The standard curve method was used to quantify unknown mRNA or miRNA abundance, and the threshold cycle (C_T) was defined as the fractional cycle number at which the fluorescence passed a fixed threshold. The amount of mRNA in each sample was normalized to the amount of GAPDH or actin mRNA, while the amounts of miRNAs were normalized to the amount of U6 small nuclear RNA as an internal control in each sample.

Western blotting. Cells were lysed in 9 M urea, 5 mM Tris-HCl (pH 6.8) and briefly sonicated. One hundred micrograms of total protein was subjected to 10% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked in 5% nonfat dry milk in phosphate-buffered saline (PBS) and then incubated with antibodies against EBNA1 (R4 rabbit serum at 1:1,000 [17] or OT1X [51] at 1:2,000), β-actin (1:1,000; catalog no. sc-1615; Santa Cruz), Dicer (1:2,000; catalog no. sc-30226; Santa Cruz), BZLF1 (1:5,000; catalog no. sc-53904; Santa Cruz), and BMRF1 (1:10,000; MAB8186; Chemicon). The blots were washed and then probed with goat antimouse peroxidase (1:3,000) or goat antirabbit peroxidase (1:5,000) (both from Santa Cruz) and then developed using enhanced chemiluminescence (ECL) reagents (PerkinElmer). Membranes were stripped in 0.1 M glycine, pH 2.9, for 30 min, washed in PBS-Tween 20, blocked briefly, and reprobed with the subsequent antibody as described above.

Immunofluorescence microscopy. Cells grown on coverslips were fixed with 3.7% formaldehyde in PBS for 20 min, rinsed twice in PBS, and permeabilized with 1% Triton X-100 in PBS for 5 min. Samples were blocked with 4% BSA in PBS, followed by incubation with primary antibodies against EBNA1 (R4 rabbit serum at a 1:400 dilution) and BZLF1 (1:600; catalog no. sc-53904; Santa Cruz) and then incubation with the secondary antibodies goat antirabbit Alexa Fluor 555 (1:800; Molecular Probes) and goat antimouse Alexa Fluor 488 (1:800; Molecular Probes) in 4% BSA. Coverslips were mounted onto slides using ProLong Gold antifade medium containing DAPI (4′,6-diamidino-2-phenylindole; Invitrogen). Images were obtained using the ×40 oil objective on a Leica inverted fluorescence microscope and processed using OpenLAB (version 4.0.2) software.

Luciferase reporter assay. Luciferase reporter assays for effects mediated by let-7a interactions with the Dicer 3′ UTR involved three plasmids: pGL3 expressing firefly luciferase (Promega), pGL3 containing a fragment of the wild-type (wt) Dicer 3′ UTR with let-7 target sites (Dicer wt UTR), and pGL3 containing the Dicer 3′ UTR in which the let-7 target sequence is mutated (Dicer mut UTR). These plasmids were kindly provided by Takashi Takahashi and have been described elsewhere (52). These constructs were each cotransfected with pc3EBNA1 or pcDNA3 (1 μg) and pRL-TK (0.2 μg; Promega), a plasmid expressing *Renilla* luciferase that was used as a control for nonspecific expression effects. Transfections were performed as described above, except that cells were plated at 2×10^5 per well. Luciferase assays were performed 24 h following transfection using a dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to *Renilla* luciferase activity for each sample, and each assay was performed in triplicate.

Transfection of miRNA inhibitors and mimics. For let-7a overexpression, 2×10^4 AGS-EBV or HONE-Akata cells were seeded in 6-well plates and immediately transfected with 50 pmol synthetic let-7a mimic or negative-control miRNA (Life Technologies) using 2 μl Lipofectamine 2000. A second round of transfection was performed 24 h later. At 24 to 48 h following the second transfection, cells were treated with 3 mM sodium butyrate (NaB) and 20 ng/ml 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to induce reactivation. Sixteen to 18 h later, floating and adherent cells were harvested and analyzed by Western blotting or immunofluorescence microscopy. For let-7 sponge experiments, 5×10^5 AGS-EBV or HONE-Akata cells in 10-cm dishes were transfected with 8 μg of the murine stem cell virus (MSCV) puro let-7 sponge plasmid (a gift from Phil Sharp [53]; Addgene plasmid 29766) or the MSCV empty-control plasmid (obtained from Lin He [54]). Forty-eight to 72 h later, EBV lytic reactivation was induced as described above for the let-7a mimic experiments.

RESULTS

Identification of cellular miRNAs whose levels are affected by EBNA1. To investigate the effects of EBNA1 on the expression of cellular miRNAs, two EBV-negative NPC cell lines (HONE1 and CNE2Z) were cotransfected with plasmids expressing YFP and either EBNA1 or empty control plasmid. In samples where the EBNA1 expression plasmid was included, YFP-positive cells were confirmed to be EBNA1 positive by immunofluorescence microscopy (data not shown). At 24 h posttransfection, YFP-positive cells were isolated by fluorescence-activated cell sorting, followed by isolation of small RNAs (18 to 26 nucleotides). These RNAs were ligated to linkers, reverse transcribed, and PCR amplified. The resultant cDNAs were subjected to high-throughput sequencing. The sequencing reads were then stripped of the 5′ and 3′ linker sequences and mapped to MiRbase 18 with high stringency; perfect matches that were at least 17 nucleotides long were considered for this analysis. Library quality and characteristics, including the size and the first nucleotide distribution of the reads, were analyzed (see Table S1 in the supplemental material). The majority of the miRNAs were 22 nucleotides long and started with nucleotide T, corresponding to features of most mature mammalian miRNAs. The recovery of individual miRNAs in each sample is shown in Table S2 in the supplemental material. The distribution of miRNA families recovered from the two cell lines (in the absence of EBNA1) is shown in Fig. 1. miR-21 and the let-7 family of miRNAs were the miRNAs expressed at the highest levels in both cell lines.

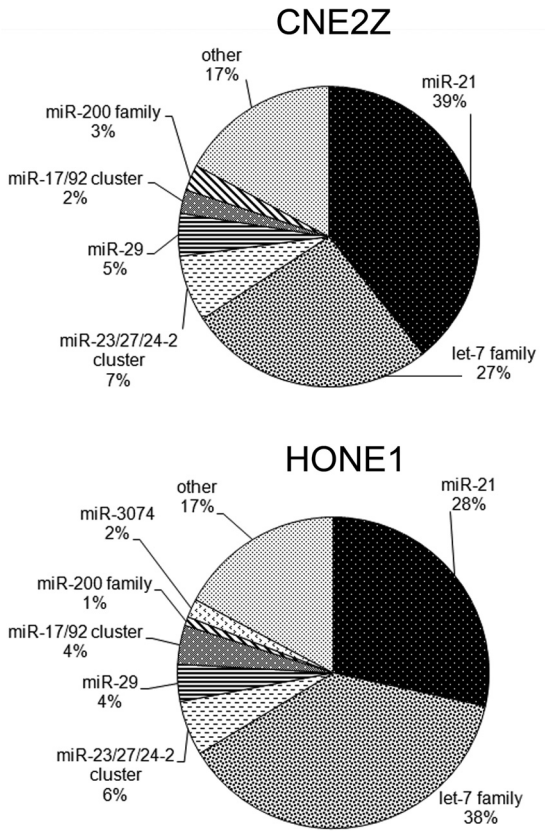

FIG 1 Abundance of miRNA families in HONE1 and CNE2Z cells. Pie charts show the general distribution of the most abundant cellular miRNA families in the HONE1 and CNE2Z cell lines.

To determine whether transient expression of EBNA1 caused any changes in cellular miRNAs, the number of reads of each miRNA were normalized to the total number of miRNAs in each sample, and this normalized miRNA level in EBNA1-expressing cells was divided by that in the control samples transfected with an empty plasmid. This ratio was determined independently for the HONE1 and CNE2Z cells. miRNAs whose level increased 1.3-fold or more or decreased 0.7-fold or less in both cell lines are shown in Table 1. By these criteria, 13 miRNAs were consistently upregulated and 6 were downregulated by EBNA1, indicating that EBNA1 affects the levels of a small subset of miRNAs. Strikingly, 7 of the 13 miRNAs that were upregulated by EBNA1 are members of the let-7 family, suggesting that EBNA1 may impact cellular processes through let-7 miRNAs. To determine if EBNA1 had global effects on miRNA levels, we calculated the percentage of recovered small RNA that mapped to miRNA for each sample (see Table S1 in the supplemental material). On average, samples with EBNA1 had slightly less miRNA (43% ± 9.7%) than samples without EBNA1 (52% ± 3.9%); however, this was not a statistically significant difference ($P = 0.07$) due to the high degree of variability in the EBNA1 samples.

Verification of effects of EBNA1 on let-7a miRNA. Initial verification of the effects of EBNA1 on specific miRNAs was conducted by generating independent RNA samples from CNE2Z and HONE1 cells after transient transfection with an EBNA1 expression or empty plasmid, followed by quantitative reverse transcription-PCR (qRT-PCR) analysis of three selected miRNAs (let-7a, let-7g, and miR-92a) using TaqMan miRNA assay kits (Fig. 2A). The values in Fig. 2 were normalized to those for RNU6 small RNA, and very similar results were obtained when values were normalized to those for RNU44 small RNA (data not shown). This analysis confirmed that let-7a, let-7g, and miR-92a miRNA levels were consistently increased by EBNA1 in both cell lines.

Further studies on the effects of EBNA1 on miRNA were focused on let-7a miRNA, since this miRNA has multiple ties to cancer and was a prominent hit for EBNA1 regulation in both cell lines. To verify the contribution of EBNA1 to let-7a regulation, we tested whether silencing of EBNA1 in EBV-positive NPC cells would lower let-7a levels. To this end, C666-1 and HONE1-Akata cells were transfected with either AllStar negative-control siRNA or siRNA that we have previously shown specifically targets EBNA1 (15, 58), and downregulation of EBNA1 was confirmed by Western blotting (Fig. 2B). The quantity of let-7a miRNA determined by qRT-PCR relative to the amount of RNU6 small RNA showed that EBNA1 depletion decreased the amount of let-7a miRNA approximately 2-fold in both cell lines.

We also examined the effect of EBNA1 on let-7a miRNA in AGS gastric carcinoma cells latently infected with EBV (AGS-EBV). Consistent with the results obtained with other cell lines, overexpression of EBNA1 (from an adenovirus delivery vector) in AGS-EBV cells or parental AGS cells increased let-7a levels relative to those observed with the negative-control adenovirus expressing β-Gal (Fig. 2C). Conversely, downregulation of EBNA1 with siRNA in AGS-EBV cells consistently resulted in an ~2-fold decrease in the amount of let-7a miRNA (Fig. 2C). The results indi-

TABLE 1 Change in miRNA regulation upon EBNA1 expression in HONE1 and CNE2Z cells[a]

Type of change in regulation and miRNA	Avg ± SD change in regulation	
	HONE1 cells	CNE2Z cells
Upregulated		
has-let-7d	3.06 ± 0.22**	3.21 ± 2.2
hsa-miR-92a-1-5p	2.64 ± 0.06***	7.06 ± 0.5***
hsa-let-7e-5p	2.64 ± 0.64*	1.45 ± 0.23*
hsa-miR-193b-3p	2.59 ± 0.008***	9.9 ± 2.56**
hsa-let-7a-5p	2.34 ± 0.27**	1.64 ± 0.1**
hsa-let-7f-5p	2.25 ± 0.22**	1.5 ± 0.23*
hsa-miR-98	2.02 ± 0.38*	1.56 ± 0.11**
hsa-miR-181a-5p	1.84 ± 0.4*	1.58 ± 0.38
hsa-miR-576-5p	1.52 ± 0.29*	2.04 ± 0.12***
hsa-let-7b-5p	1.51 ± 0.17*	1.51 ± 0.38
hsa-miR-26a-5p	1.51 ± 0.19*	1.34 ± 0.04**
hsa-let-7g-5p	1.5 ± 0.39	1.95 ± 0.22**
hsa-miR-320a	1.44 ± 0.02***	1.35 ± 0.21
Downregulated		
hsa-miR-15b-5p	0.48 ± 0.33	0.76 ± 0.06**
hsa-miR-151a-3p	0.43 ± 0.05***	0.37 ± 0.02***
hsa-miR-3613-5p	0.4 ± 0.17**	0.42 ± 0.23***
hsa-miR-27b-3p	0.4 ± 0.11**	0.79 ± 0.04***
hsa-miR-744-5p	0.35 ± 0.18**	0.59 ± 0.102**
hsa-miR-27a-3p	0.15 ± 0.04***	0.84 ± 0.003***

[a] The average fold change in the expression of cellular miRNAs relative to the total cellular miRNA count upon EBNA1 overexpression in HONE1 and CNE2Z cells. The average fold changes and standard deviations from two biologically independent experiments are shown. let-7 family miRNAs are shaded gray. *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.

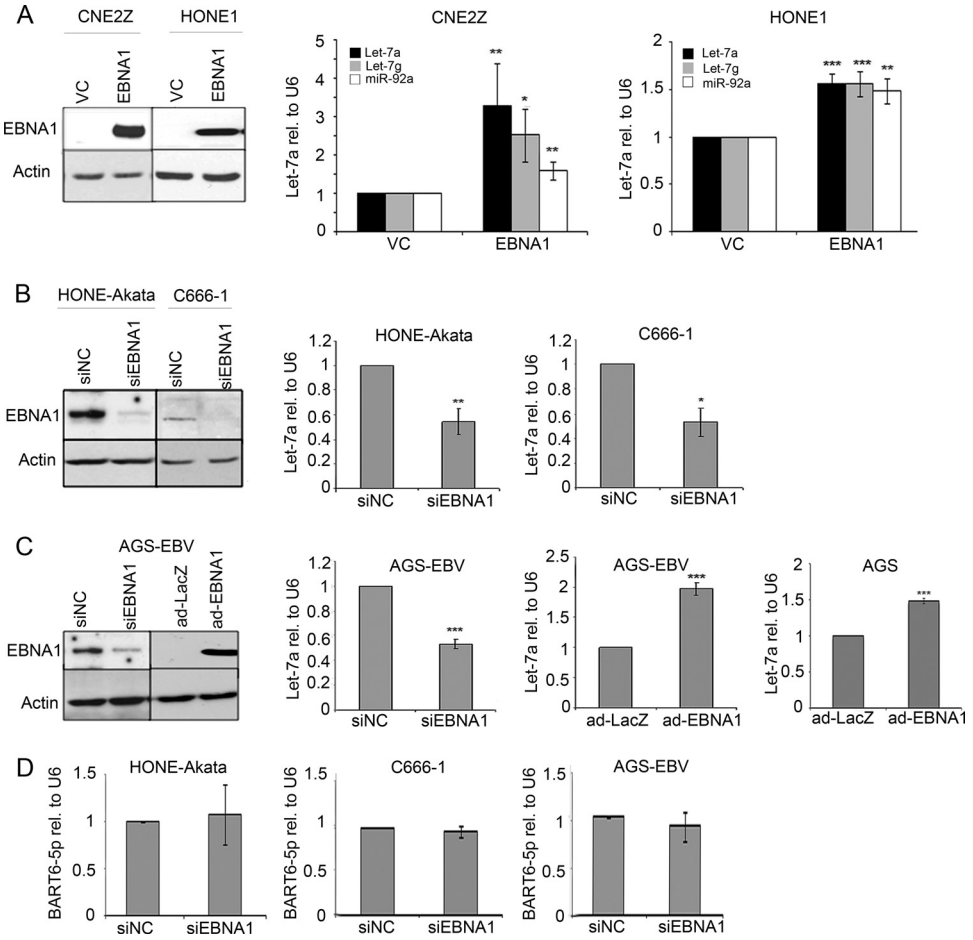

FIG 2 EBNA1 induces let-7a miRNA. (A) CNE2Z and HONE1 cells were transfected with a plasmid expressing EBNA1 or an empty plasmid vector control (VC), and then samples were processed for Western blotting (left) or for qRT-PCR quantification of let-7a, let-7g, or miR-92a miRNA (bar graphs). miRNA values were normalized to those for U6 RNA. Bar graphs show average values with standard deviations from three independent experiments in which values for the vector control were set equal to 1. (B) C666-1 and HONE-Akata cells were transfected with siRNA targeting EBNA1 (siEBNA1) or AllStar negative-control siRNA (siNC), and samples were processed as described in the legend to panel A. The values shown in the bar graphs are relative to the value for the negative-control siRNA, which was set equal to 1. (C) AGS-EBV cells were treated with siRNA targeting EBNA1, AllStar negative-control siRNA, an adenovirus vector expressing FLAG-tagged EBNA1 (ad-EBNA1), or a negative-control adenovirus vector expressing β-Gal (ad-LacZ), and samples were processed as described in the legend to panel A. Results of experiments in which AGS cells were treated with adenovirus vector expressing FLAG-tagged EBNA1 or a negative-control adenovirus vector expressing β-Gal are also shown (right). let-7a levels in EBNA1 overexpression experiments relative to those for the LacZ negative-control adenovirus are shown. (D) C666-1, HONE-Akata, and AGS-EBV cells were transfected with siRNA targeting EBNA1 or AllStar negative-control siRNA, and samples were processed for qRT-PCR quantification of BART6-5p. BART6-5p values were normalized to those for U6 RNA. *P* values were calculated and are indicated as follows: *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.

cate that EBNA1 upregulates let-7a miRNA in both NPC and gastric carcinoma cells.

Since EBNA1 has a role in maintaining EBV genomes, we also examined the effect of EBNA1 silencing in C666-1, HONE1-Akata, and AGS-EBV cells on an miRNA of EBV (BART6-5p) as an indicator of the presence of the EBV genomes (Fig. 2D). BART6-5p levels were not affected by EBNA1 silencing in any cell line, indicating that the decrease in let-7a observed upon EBNA1 silencing is not due to the loss of the EBV genome.

EBNA1 induces let-7a by increasing primary transcripts. Expression of miRNAs can be regulated at transcriptional and post-transcriptional levels. let-7a miRNA is derived from primary transcripts expressed from three different chromosomal locations; *pri-let-7afd* on chromosome 9, *pri-let-7a-2* on chromosome 11, and *pri-let-7a-3* on chromosome 22. We used qRT-PCR to measure possible changes in the levels of these transcripts when EBNA1 was expressed in the EBV-negative HONE1 cells or silenced in EBV-positive HONE1-Akata cells. While *pri-let-7a-3* transcripts were barely detectable in these cells, *pri-let-7afd* and *pri-let-7a-2* transcripts were consistently detected and their levels were compared after normalization to the level of GAPDH mRNA in each sample. EBNA1 expression in HONE1 cells resulted in consistent increases in both *pri-let-7afd* and *pri-let-7a-2* transcript levels (Fig. 3A). Similar results were obtained when the data were normalized to the level of β-actin mRNA or 18S rRNA (data not shown). Conversely, EBNA1 depletion in HONE-Akata cells resulted in decreased levels of both *pri-let-7afd* and *pri-let-7a-2* transcripts (Fig. 3B). Therefore, EBNA1 appears to increase the levels of mature let-7a miRNA by inducing the expression of let-7a primary transcripts.

EBNA1 is known to transactivate the expression of EBV latency genes and may also induce the expression of some cellular genes.

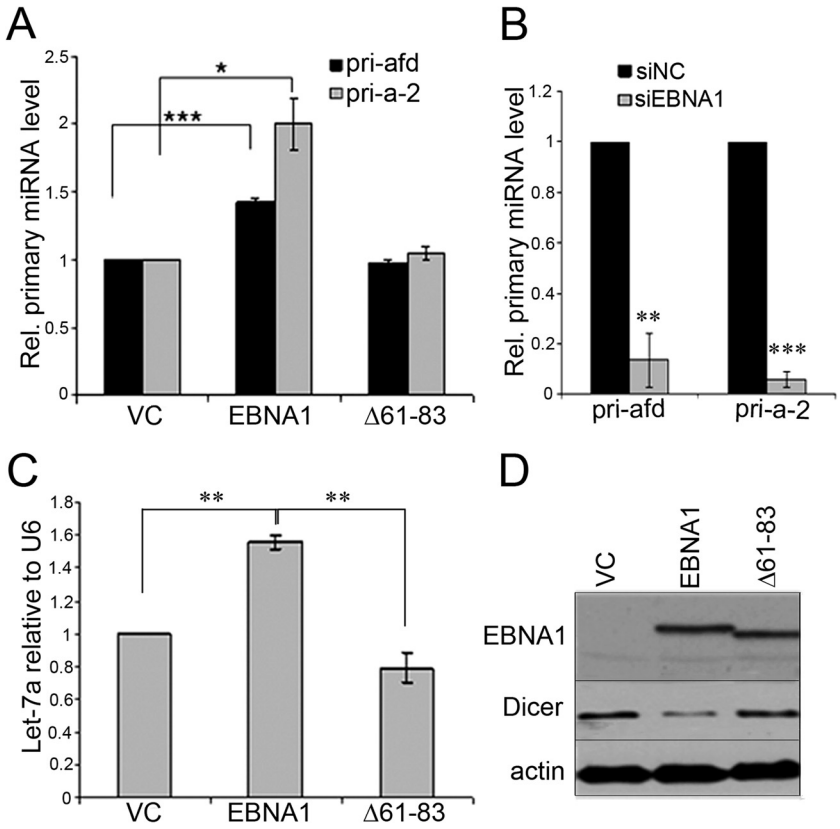

FIG 3 EBNA1 induces let-7a primary transcripts. (A) HONE1 cells were transfected with plasmids expressing EBNA1, the Δ61-83 EBNA1 mutant, or the empty plasmid vector control (VC), and expression of *pri-let-7afd* (pri-afd) and *pri-let-7a-2* (pri-a-2) relative to that of GAPDH mRNA was quantified by qRT-PCR. (B) HONE-Akata cells were transfected with siRNA targeting EBNA1 (siEBNA1) or AllStar negative-control siRNA (siNC), and then *pri-let-7afd* and *pri-let-7a-2* transcript levels were determined as described in the legend to panel A. (C) Mature let-7a miRNA from HONE1 cells transfected with EBNA1 or EBNA1 Δ61-83 mutant expression plasmid or empty plasmid was quantified by qRT-PCR. Values were normalized to those for RNU6 small RNA. All bar graphs show average values with standard deviations calculated from three independent experiments relative to the value for the empty vector control or negative-control siRNA, which was set equal to 1. (D) HONE1 cells transfected as described in the legends to panels A and C were analyzed by Western blotting using antibodies specific to Dicer, EBNA1, and actin (loading control). *P* values were calculated and are indicated as follows: *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.

The transcriptional activation function of EBNA1 can be specifically disrupted by deletion of amino acids 61 to 83 (Δ61-83) without affecting the replication or segregation functions of EBNA1 (11). We used the EBNA1 Δ61-83 mutant to determine if the EBNA1-induced increase in primary let-7a transcripts involves the transactivation activity of EBNA1. Comparisons of the *pri-let-7afd* and *pri-let-7a-2* levels after expression of EBNA1 or the Δ61-83 mutant in HONE1 cells indicated that loss of the sequence from amino acids 61 to 83 abrogated the ability of EBNA1 to induce these transcripts (Fig. 3A). In addition, we compared the ability of EBNA1 and the Δ61-83 mutant to induce mature let-7a miRNA and found that this effect of EBNA1 was also dependent on the transactivation region from amino acids 61 to 83 (Fig. 3C). Importantly, EBNA1 and the Δ61-83 mutant were expressed at similar levels (Fig. 3D), indicating that the lack of an effect of the Δ61-83 mutant was not due to insufficient expression. The results as a whole suggest that the increased levels of let-7a miRNA seen in the presence of EBNA1 are due to the ability of EBNA1 to transactivate the expression of let-7a primary transcripts.

EBNA1 negatively regulates Dicer through let-7a miRNA. let-7 miRNA has been shown to regulate Dicer protein expression by binding to multiple target sites in the 3′ UTR and additional target sites in the coding sequences of the Dicer mRNA (52, 55). let-7a miRNA has also been shown to decrease Dicer mRNA levels in some cell lines (56). To further evaluate the significance of the EBNA1-induced changes in let-7a miRNA levels, we investigated whether EBNA1 affected the levels of Dicer protein and mRNA. Transient expression of EBNA1 in HONE1 and CNE2Z cells was found to decrease both Dicer protein (Fig. 4A) and mRNA (Fig. 4B) levels. Conversely, downregulation of EBNA1 with siRNA in EBV-positive AGS-EBV and C666-1 cells caused an increase in Dicer protein and mRNA levels relative to those after negative-control siRNA treatments (Fig. 4C and D). The EBV BART6-5p miRNA was previously reported to target Dicer (57), but since EBNA1 depletion did not affect BART6-5p levels (Fig. 2D), the effect of EBNA1 on Dicer is not mediated by BART6-5p. The results are consistent with the possibility that EBNA1 regulates Dicer expression by altering let-7a miRNA levels. This possibility was further supported by the finding that the EBNA1 Δ61-83 mutant, which did not affect let-7a levels, also did not affect Dicer levels (Fig. 3D).

To more conclusively determine if EBNA1 affects Dicer due to changes in let-7 miRNA levels, we performed a luciferase reporter assay. The assay involved three constructs: one containing the wild-type (wt) Dicer 3′ UTR sequence downstream of the luciferase gene, a second one in which the 3′ UTR is mutated in all three

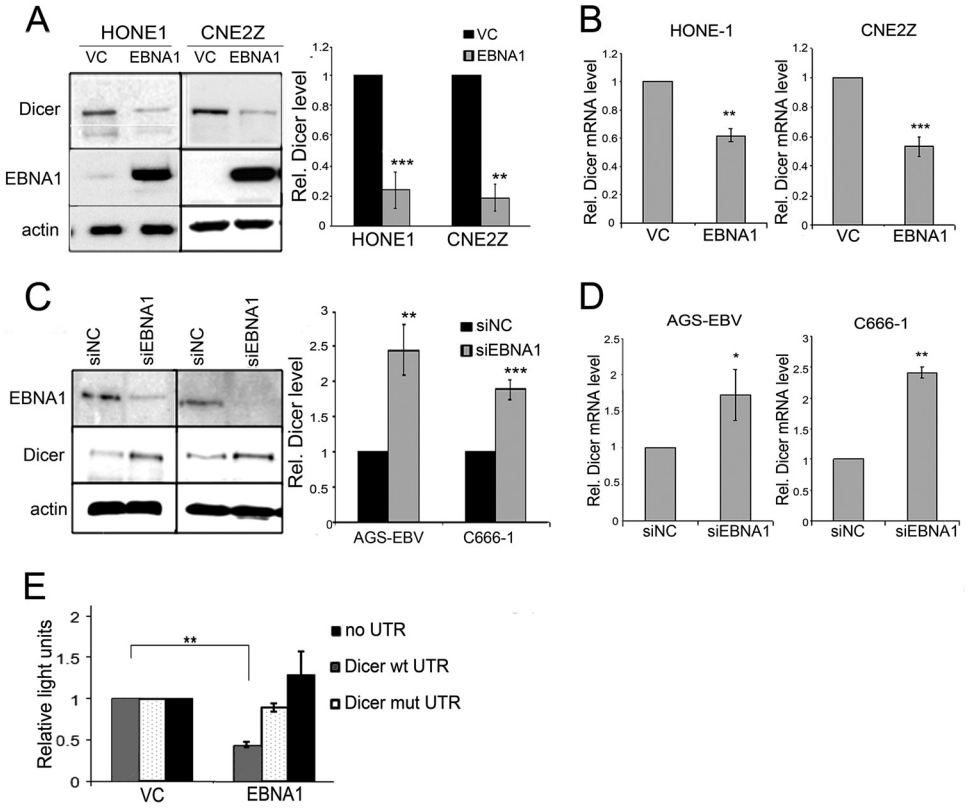

FIG 4 Effect of EBNA1 on Dicer. EBNA1 was transiently expressed in HONE1 and CNE2Z cells (A and B) or downregulated with siRNA in AGS-EBV and C666-1 cells (C and D). Dicer protein levels were then examined by Western blotting, and quantification of Dicer bands (A and C) and Dicer mRNA levels relative to those for GAPDH mRNA (B and D) were determined by qRT-PCR. Average values from three experiments relative to those for the empty vector control (VC) or AllStar negative-control siRNA (siNC), which were set equal to 1, are shown. (E) HONE1 cells were transfected with the EBNA1 expression plasmid or an empty plasmid, along with a luciferase reporter construct containing either the wt Dicer 3′ UTR, the Dicer 3′ UTR with mutated let-7 binding sites, or no UTR, as indicated. Luciferase expression was quantified at 24 h posttransfection. Average values from three independent experiments are shown relative to the value for an empty plasmid control with a luciferase reporter lacking a UTR, which was set equal to 1. *P* values were calculated and are indicated as follows: *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$; ***, $P < 0.001$.

let-7a binding sites, and a third one lacking a 3′ UTR (52). HONE1 cells were transfected with each of these reporter plasmids and either the EBNA1 expression plasmid or an empty plasmid, and then luciferase expression was measured. EBNA1 was consistently found to decrease the expression of luciferase in the construct containing the wt UTR relative to that in the empty plasmid control (Fig. 4E). However, EBNA1 did not affect luciferase expression in the reporter plasmid containing the mutated UTR and slightly increased luciferase expression when no UTR was present (Fig. 4E). As expected, in the absence of EBNA1, luciferase expression was similar for the constructs. The results indicate that the effect of EBNA1 on Dicer levels requires let-7 interactions with the Dicer UTR and therefore support the hypothesis that EBNA1 affects Dicer due to modulation of let-7 miRNA.

let-7a suppresses EBV lytic reactivation. It was previously reported that Dicer silencing or downregulation by the Dicer-targeted EBV miRNA BART6-5p resulted in decreased expression of the BZLF1 and BRLF1 lytic switch proteins, suggesting that Dicer positively contributes to EBV reactivation of the lytic cycle (57). Therefore, we examined whether let-7a miRNA influenced EBV reactivation through its effects on Dicer. To this end, HONE-Akata cells were transfected with a let-7a miRNA mimic or negative-control miRNA, followed by treatment with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and sodium butyrate (NaB) to induce BZLF1 expression and EBV reactivation. As expected, overexpression of let-7a decreased Dicer levels (Fig. 5A). In addition, a corresponding decrease in BZLF1 expression levels as well as in the percentage of BZLF1-positive cells was observed (Fig. 5A and B).

We also repeated these experiments using a let-7 sponge construct that expresses RNA containing multiple binding sites for the let-7 miRNA family members and has been shown to inhibit the function of most let-7 miRNAs (53). Expression of the let-7 sponge in HONE-Akata cells was found to increase the levels of expression of both Dicer and BZLF1 (Fig. 5C) as well as increase the percentage of cells expressing BZLF1 (Fig. 5D).

To ensure that the effect of let-7a and Dicer on lytic reactivation was not specific to HONE-Akata cells, we repeated the let-7a mimic and sponge experiments in AGS-EBV cells. These cells have a low level of spontaneous reactivation and therefore provided the opportunity to observe the effects on spontaneous BZLF1 expression (in the absence of TPA-NaB treatment). Treatment with the let-7a mimic decreased the expression of both Dicer and BZLF1 both before and after induction of reactivation by TPA-NaB (Fig. 5E). Similarly, the EBV lytic replication protein BMRF1, which is induced by TPA-NaB treatment, was also found to be decreased

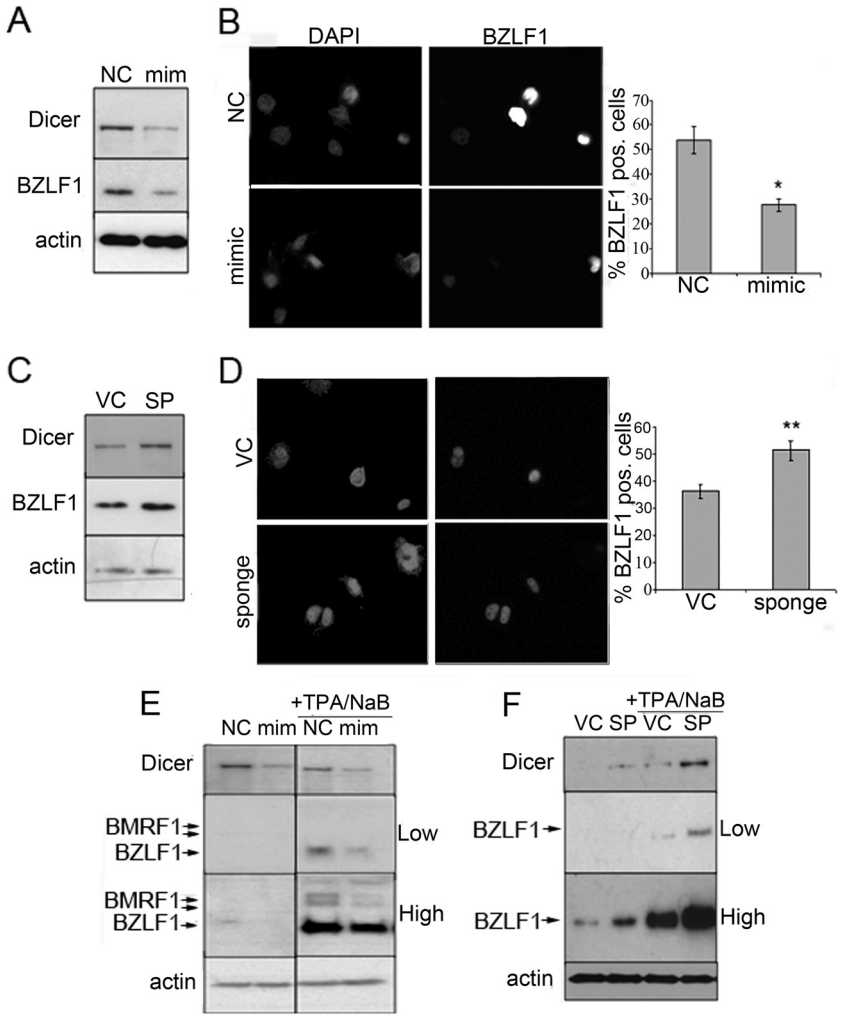

FIG 5 let-7a suppresses EBV reactivation. (A to D) HONE-Akata cells were transfected with a synthetic let-7a mimic (mim) or negative-control miRNA (NC) (A and B) or with a plasmid expressing let-7 sponge mRNA or the vector control (VC) (C and D) and then treated with TPA/NaB. Dicer and BZLF-1 expression were then analyzed by Western blotting (A and C). In addition, immunofluorescence microscopy was performed with BZLF1 antibody, and the percentage of BZLF-1-positive (pos.) cells was determined (B and D). (E and F) AGS-EBV cells were transfected with the let-7a mimic or negative-control miRNA (E) or with the plasmid expressing let-7 sponge mRNA or the vector control (F). At 48 h posttransfection, cells were treated with TPA and NaB or left untreated, and then Western blotting assays were performed on cell lysates using antibodies specific for Dicer, BZLF1, BMRF1, and actin (loading control). Two different exposures of the BZLF1 blot are shown (light and dark). *P* values were calculated and are indicated as follows: *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$.

by the let-7a mimic. Conversely, expression of the let-7 sponge increased the levels of Dicer and BZLF1 both before and after TPA-NaB treatment (Fig. 5F). The results indicate that let-7a suppresses EBV reactivation and suggests that our previous observation that EBNA1 can suppress EBV reactivation (58) may be due to its effects on let-7a.

DISCUSSION

EBNA1 is a key protein in EBV latency, playing multiple important roles in the maintenance and gene expression of EBV episomes through interactions with specific DNA sequences in the EBV genome. In addition, EBNA1 impacts cellular processes through interactions with specific host proteins, likely contributing to cell survival in latency and oncogenesis (20–22, 59). EBNA1 has also been found to interact with some RNA molecules (60, 61). In recent years, miRNAs have emerged as important regulators of many cellular processes that impact tumorigenesis and viral infections, but the potential effects of EBNA1 on most miRNAs had not been determined. Here we used small RNA cloning coupled with high-throughput sequencing to investigate the effects of EBNA1 on cellular miRNAs in the context of NPC cells and identified several miRNAs that are consistently up- or downregulated by EBNA1 in two different NPC cell lines.

Approximately half of the miRNAs that were upregulated by EBNA1 belonged to the let-7 family. The let-7 family of miRNAs is very prevalent in both NPC cells lines, second only to miR-21 miRNAs (which were not affected by EBNA1). let-7 microRNAs were first discovered in *Caenorhabditis elegans* (62), are highly conserved in species ranging from worms to humans, and have conserved mRNA targets (63). In humans, the let-7 family contains 13 members, transcribed from nine different locations in the genome (64). The members of the let-7 family of miRNAs are considered tumor suppressors, due to their ability to downregulate the expression of multiple oncogenes (65, 66) and the fact that

they are often downregulated in human cancers (67–69). However, increased expression of let-7a-3 in cancer cells has also been observed (70), suggesting that let-7 miRNAs do not always play a tumor-suppressive role.

In addition to effects on let-7 miRNA, EBNA1 caused a prominent increase in the levels of miR-92a and miR-193b-3p in both CNE2Z and HONE1 NPC cell lines. The contribution of miR-193b to tumorigenesis is currently unclear, as it has been reported to have both oncogenic and tumor-suppressive properties (71–73). miR-92a is part of the miR-17/92 cluster, a group of 6 mature miRNAs expressed from a common polycistronic primary transcript (74–76). The miR-17/92 genomic location is frequently amplified in human lymphomas and solid tumors, and overexpression of miR-92a can confer oncogenic properties (74–77). Therefore, the upregulation of miR-92a by EBNA1 could be a factor contributing to NPC.

One of the let-7 miRNAs that we found to be upregulated by EBNA1 was let-7a. let-7a induction in response to EBNA1 expression was verified in multiple EBV-negative carcinoma cell lines, while EBNA1 silencing in multiple EBV-positive carcinoma cell lines was found to decrease let-7a levels. The two prevalent let-7a primary transcripts in NPC cells (*pri-let-7afd* and *pri-let-7a-2*) were both induced by EBNA1, but neither of these primary transcripts nor mature let-7a was induced by EBNA1 lacking the transactivation sequence (the EBNA1 Δ61-83 mutant), suggesting that EBNA1 upregulates let-7a by inducing the transcription of its pri-RNAs. In addition to generating let-7a, *pri-let-7a-2* and *pri-let-7afd* also generate let-7d, let-7f, and miR-100 mature miRNAs (78, 79). While miR-100 was not reliably detected in our samples, the EBNA1-induced increase in let-7d and let-7f could be explained by the increase in *pri-let-7afd*. Little is known about the DNA sequences and proteins that control the expression of *pri-let-7afd* and *pri-let-7a-2*. There are several scenarios by which EBNA1 may induce the expression of these primary RNAs, including (i) binding directly to promoter or enhancer sequences controlling these genes, (ii) being recruited to the promoter/enhancer sequences for these genes through interactions with cellular proteins, and (iii) inducing the expression of a cellular protein that in turn induces the expression of *pri-let-7afd* and *pri-let-7a-2*. Analysis of the 50-kb sequence upstream of the putative transcription start sites for *pri-let-7afd* and *pri-let-7a-2* failed to reveal any sequence resembling a consensus EBNA1 recognition site (data not shown).

let-7 miRNAs share a large number of target mRNAs due to the similarity of their seed sequences located from nucleotides 2 to 8 at the 5′ end of the miRNA. These targets include Myc, Ras, HmgA2, and Dicer (52, 65, 80), all of which we investigated for putative regulation by EBNA1. Myc and Ras were not found to be decreased by EBNA1 expression in EBV-negative cells, although some increase of Ras after EBNA1 silencing in EBV-positive cells was observed (data not shown). HmgA2 was upregulated upon EBNA1 silencing in C666-1 cells but was not expressed in any of the other NPC or AGS cell lines that we examined (data not shown). Dicer was the only let-7 target that we found to reproducibly decrease upon expression of EBNA1 and to increase upon EBNA1 silencing in EBV-positive cells. Dicer mRNA contains a large (4-kb) 3′ UTR with target sites for multiple miRNAs, including all let-7 members and miR-103/107 (81). The results of the reporter assays with the Dicer 3′ UTR and mutated UTR lacking let-7 binding sites indicate that the EBNA1-induced decrease in Dicer levels involves let-7. Although Dicer is known to play a major role in processing most cellular miRNAs, downregulation of Dicer by EBNA1 did not result in a uniform decrease in the level of miRNAs. Instead, a small subset of miRNAs was preferentially up- or downregulated, suggesting that the reduction in the Dicer protein level was insufficient to affect miRNA processing in general. In keeping with this observation, the level of total miRNA recovered with and without EBNA1 was not significantly different.

A previous report on the EBV-encoded BART6 miRNA identified Dicer to be one of its targets and presented data that this downregulation of Dicer resulted in decreased expression of some EBV mRNAs, including mRNA for the BZLF1 and BRLF1 proteins that initiate lytic infection (57). Here we have shown that let-7a, which downregulates Dicer, also decreases the expression of BZLF1. Specifically, both spontaneous BZLF1 expression in the permissive AGS-EBV cells and chemically induced BZLF1 expression in HONE-Akata and AGS-EBV cells were decreased by expression of a let-7a mimic and increased by expression of a let-7 sponge, suggesting that let-7a helps to maintain latency in EBV-infected cells. Since EBNA1 upregulates let-7a, EBNA1 would also be expected to contribute to the maintenance of EBV latency. Recently, we found that EBNA1 affects the balance between EBV latent and lytic infection in epithelial cells in two general ways: in latent infection, EBNA1 suppresses spontaneous reactivation, whereas after the switch to the lytic cycle has occurred (e.g., by chemical induction), EBNA1 positively contributes to efficient lytic infection (58). The latter contribution of EBNA1 was shown to involve the EBNA1-induced loss of PML nuclear bodies, since this effect of EBNA1 was lost in PML-negative cells. However, the mechanism by which EBNA1 promoted latency had not been determined, other than showing that it was independent of PML nuclear bodies (58). Our current observation that EBNA1 induces let-7a miRNA, which in turn represses reactivation, provides an explanation of how EBNA1 could promote EBV latency.

Three other miRNAs, BART6-5p, miR-200b and miR-429, have previously been reported to affect EBV reactivation. BART6-5p is an miRNA of EBV that downregulates Dicer, leading to decreased expression of the lytic switch proteins BZLF1 and BRLF1 (57). We have shown that EBNA1 depletion does not affect BART6-5p levels, suggesting that the effects of EBNA1 on latency are independent of BART6. However, it is interesting that both BART6 and EBNA1 appear to promote latency by lowering Dicer expression. miR-200b and miR-429 can induce EBV reactivation by downregulating ZEB1 and ZEB2, two host proteins that suppress BZLF1 expression by binding its promoter (39). Neither miR-200b nor miR-429 was consistently affected by EBNA1 (see Table S2 in the supplemental material), and neither ZEB1 nor ZEB2 is expressed in AGS cells (82), suggesting that the effects of EBNA1 and let-7a on EBV reactivation that we have observed are independent of ZEB1 and ZEB2.

A recent study on the effect of EBV infection on miRNAs in AGS cells found that the levels of many miRNAs associated with tumor suppression, including let-7 family members, were decreased by EBV infection (83). The fact that we have shown that expression of EBNA1 on its own increases let-7 miRNA levels most likely indicates that another component of the EBV genome (other than EBNA1) must be responsible for decreasing let-7 miRNA levels. Marquitz et al. (83) came to the same conclusion when they examined EBNA1 expression on its own in AGS cells and concluded that EBNA1 did not decrease let-7a or let-7b

miRNA levels but, rather, slightly increased let-7a and let-7b miRNA levels. We also found that EBNA1 expression on its own subtly increases let-7a expression in AGS cells but that the effect of EBNA1 modulation on let-7a is more pronounced in CNE2, HONE1, AGS-EBV, HONE-Akata, and C666-1 cells.

It is also interesting to consider how the partial downregulation of Dicer by EBNA1 might contribute to EBV-induced cancers. It was recently reported that Dicer mRNA levels are decreased in most cervical cancer samples relative to the levels in normal tissue and that lower Dicer levels are associated with tumor progression and metastasis in cervical cancer (84). In another study, partial downregulation of Dicer by miR-103/107 was found to increase cell migration and promote the epithelial-to-mesenchymal transition (EMT) (81). Interestingly, EBNA1 was recently shown to induce the EMT in CNE1 and CNE2 NPC cell lines, and high levels of EBNA1 expression in NPC tissue samples were found to be associated with lymph node metastasis (85). The authors suggested that the small decrease in miR-200b levels seen in CNE1 cells expressing EBNA1 might account for the EBNA1 induction of the EMT (through effects on ZEB1 and ZEB2). Our results, combined with the reported effects of Dicer on EMT, suggest that EBNA1 might also affect EMT and metastasis by downregulating Dicer.

In summary, we have shown that EBNA1 can affect the level of specific cellular miRNAs in NPC cells, including the upregulation of multiple let-7 miRNAs. In addition, our data indicate that induction of let-7a by EBNA1 promotes latency through effects on Dicer. How other EBNA1-regulated miRNAs affect EBV infection and associated cancers remains to be determined.

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